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Patents ADP number *(if you know it)* 00798207001

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4. Title of the invention — Use of Peptides

5. Name of your agent *(if you have one)* — D YOUNG & CO

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USE OF PEPTIDES

The present invention relates to eukaryotic Initiation Factor 4G (eIF4GI, GII) and derivative s of eIF4E Binding Proteins (4E-BP1, 2, 3, 4) that interact with it.

By way of introduction, the proposed mechanism of eukaryotic initiation factor complex formation will be described with reference to Figure 1. The eIF4F complex is capable of initiating translation of 5' capped (m^7G) mRNAs[1]. This complex comprises eIF3, eIF4A, eIF4E and eIF4G (Fig. 1).

- eIF4G acts as a scaffold around which the other components are assembled.

- eIF4A is a helicase which is required to unwind regions of secondary structure in the 5' UTR of the mRNA.

- eIF3 is responsible for recruiting the 40S ribosomal sub-unit to the complex, interacting with both the 40S ribosomal sub-unit and eIF4G.

eIF4E binds to both eIF4G and to the m^7G cap at the 5' end of the mRNA, hence - recruiting the 40S ribosomal sub-unit to the 5' untranslated region (UTR) of capped mRNAs.

eIF4E independent routes exist for the initiation of translation of some messages[2] (e.g. via an internal ribosome entry site (IRES)) however mRNAs containing a long 5' UTR are dependent on eIF4E for the recruitment of the eIF4F complex to the m^7G cap, and the subsequent unwinding of the UTR by eIF4A. The critical role of eIF4E in cap dependent translation is attributed to the limited availability of the active species. eIF4E appears to be in limiting amounts relative to other eIF4F components[1], requires phosphorylation (by Mnk 1[3])for maximum activity and can be excluded from the eIF4F complex by binding to a 4E-BP[4,5] (Fig. 2).

There is increasing evidence for a role of eIF4E in carcinogenesis. eIF4E induces cap dependent translation initiation in response to a number of mitogenic or proliferative

stimuli[1,4,6]. Hormone and growth factor induced signal transduction can lead to hyperphosphorylation of 4E-BP by mTOR, resulting in the release of 4E-BP-bound eIF4E (Fig. 2). Similar stimuli also lead to activation of eIF4E via phosphorylation by Mnk-1. The resultant increase in eIF4E activity is required for the translation of several cap-dependent transcripts whose translation products are required for proliferation (e.g. cyclin D1[7], Ornithine Decarboxylase (ODC)[8]).

The number of reports of increased levels of eIF4E in tumour samples is growing steadily[9,10], and in some cases eIF4E levels has been proposed to be a good indicator of prognosis[11,12]. Overexpression of eIF4E in cultured cell lines is reported to result in a transformed phenotype[13,14].

Overall these results have suggested that inhibiting eIF4E would result in inhibition of cap-dependent translation, resulting in little or no expression of mRNAs with strong eIF4E dependency for translation. This is expected to cause reduction in expression of several proteins involved in proliferation, and to reduce the transformed phenotype of some tumour cells.

It has also been reported that overexpression of eIF4E is capable of acting as an anti-apoptotic survival signal in fibroblasts undergoing Myc-induced apoptosis in serum-restricted conditions[15].

Protein Microdisection

The variety of eIF4E interacting proteins (eIF4G and 4E-BPs) has allowed identification of a common motif, (K/R)xxYDRxFL(L/M), required for binding to eIF4E[4]. Subsequently a 20 amino acid fragment of human 4E-BP1 containing this motif was shown to be capable of binding to recombinant mouse eIF4E and inhibiting cap-dependant translation in an *in vitro* translation assay[16], presumably by disrupting the formation of the eIF4F complex.

The proposed approach was to use eIF4E-binding peptides (derived from eIF4G and 4E-BPs) to inhibit formation of the eIF4F complex and reduce cap-dependent translation (Fig. 3).

The present invention is based upon the observation that eIF4E binding peptides have been shown for the first time to induce programmed cell death. This observation is surprising given that the expected effect of such peptides was to reduce expression of several proteins involved in proliferation, resulting in growth inhibition of, or increased cytotoxicity to tumour cells. This surprising observation renders these peptides of utility in therapy.

The present invention therefore relates to the use of eIF4E binding peptides in the induction of programmed cell death. The particular peptides found to be capable of inducing programmed cell death include a sequence of human eIF4G$_{413-424}$, wheat eIF4G$_{62-73}$ and human eIF4E-BP$_{37-48}$ and derivatives and fragments thereof.

The peptides of use in the present invention include;

human eIF4G$_{413-424}$,	KKRYDREFLLGF
wheat eIF4G$_{62-73}$	RVRYSRDQLLDL and,
human eIF4E-BP$_{37-48}$	RIIYDRKFLLDR.

The invention also relates to the use of fragments and derivatives of these peptides. Fragments are defined herein as any portion of the peptides described that retain the activity of the parent peptide. Derivatives are defined as any modified forms of said peptides. Such derivatives may take the form of amino acid substitutions which may be in the form of like for like e.g. a polar amino acid residue for another polar residue or like for non-like e.g. substitution of a polar amino acid residue for a non-polar residue as discussed in more detail below.

In one embodiment the replacement amino acid residue is selected from the residues of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The replacement amino acid residue may additionally be selected from unnatural amino acids. Within the above definitions of the peptide carrier moieties of the present invention, the specific amino acid residues of the peptide may be modified in such a manner that retains their ability

to induce programmed cell death, such modified peptides are referred to as "variants". Thus, homologous substitution may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (O), diaminobutyric acid (B), norleucine (N), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine. Within each peptide carrier moiety more than one amino acid residue may be modified at a time, but preferably when the replacing amino acid residue is alanine, less than 3.

As used herein, amino acids are classified according to the following classes;

basic; H, K, R
acidic; D, E
polar; A, F, G, I, L, M, P, V, W
non-polar; C, N, Q, S, T, Y,
(using the internationally accepted amino acid single letter codes)
and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to substitution from within the same class, whereas non-homologous substitution refers to substitution from a different class or by an unnatural amino acid.

Examples;

Abbreviations.

Amino acid and peptide nomenclature conforms to IUPAC-IUB rules (Eur. J. Biochem. 1984, 138, 9-37). Other abbreviations: Ahx, 6-aminohexanoyl; APase, alkaline phosphatase. DE MALDI-TOF MS, delayed-extraction matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. DIEA, N,N-diisopropylethylamine. PBS, phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4); PyBOP, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reveresed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

1.1: Materials and Methods

General

The peptide deprotection / cleavage mixture used throughout was as follows: 0.75 : 0.5 : 0.5 : 0.25 : 10 (w / v / v / v / v) PhOH, H_2O, PhSMe, 1,2-ethanedithiol, TFA (Beavis, R.C.et al.,. (1992) Organic Mass Spectrometry 27, 156-158). Analytical and preparative RP-HPLC was performed using Vydac 218TP54 (4.6 x 250 mm) and 218TP1022 (22 x 250 mm) columns, respectively. Flow rates of 1 mL/min for analytical runs and 9 mL/min for preparative work were used (at 25 °C). Gradient elution with increasing amounts of MeCN in H_2O (containing 0.1 % TFA) over 20 min (anal.) and 40 min (prep.) was performed. Eluants were monitored at λ = 200 – 300 nm. Peptide samples were also analysed by DE MALDI-TOF mass spectrometry (ThermoBioAnalysis Dynamo instrument). An α-cyano-4-hydroxycinnamic acid matrix (Beavis, R.C. et al., (1992) Organic Mass Spectrometry 27, 156-158) was used and the appropriate m/*z* range was calibrated using authentic peptide standards in the m/*z* range 1,000 – 2,600.

1.2: Simultaneous multiple synthesis of peptides 21 - 36

Peptides were synthesised using a Multipin Peptide Synthesis Kit (Chiron Technologies Pty. Ltd., Clayton, VIC, Australia). Peptide chains were assembled on "Macro Crowns" (SynPhase HM Series I, Rink Amide Linker; 5.3 μmol/crown) using Fmoc-amino acids (100 mM in DMF) and PyBOP / HOBt) / DIEA (1 : 1 : 1.5) coupling chemistry. The amino acid side-chain protecting groups were 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Arg), trityl (Asn and Gln), and *t*-butyloxycarbonyl (Lys and Trp). Activated amino acid solutions were dispensed using a PinAID device (Chiron Technologies). Coupling reactions were allowed to proceed for a minimum of 4 h. All other chain assembly manipulations, including repetitive deprotection reactions (20 % piperidine in DMF) and washing cycles (DMF and MeOH), were carried out according to procedures set out in the kit manual. After coupling and deprotection of the *N*-terminal βAla residues, (+)-biotin (300 mM in DMF) was coupled (chemistry as above for amino acids) during 4 h. After washing and drying, the "Macro Crowns" were removed from the synthesis device and placed into 10-mL capped polypropylene tubes. To each tube was added 1.5 mL of cleavage /

deprotection mixture. After 2 h, the "Macro Crowns" were removed and washed with 0.5 mL each of neat TFA. To each tube containing the combined cleavage mixtures and washings Et_2O (8 mL) was added. After cooling to 4 °C, the precipitated peptides were collected by centrifugation (4 min at 5,000 r.p.m.) and decantation. The pellets were resuspended in Et_2O (5 mL / tube). The suspensions were again cooled and the peptides isolated as before. The washing process was repeated once more before the crude peptides were dried *in vacuo*.

The crude peptides were redissolved in 0.1 % aq TFA using sonication (2 mL / sample) and were applied to primed (MeOH then 0.1 % aq TFA) solid-phase extraction cartridges (Merck LiChrolut RP-18, 500 mg). These were successively washed (2 x 2 mL 0.1 % aq TFA each) and eluted (2 mL 0.1 % TFA in 6 : 4 MeCN / H_2O). The eluates were evaporated to dryness by vacuum centrifugation.

Summary of Results

<u>eIF4G peptides bind to recombinant human eIF4E *in vitro*.</u>

1) Human eIF4G$_{(413-424)}$-Penetratin* binds recombinant human eIF4E *in vitro* (Fig. 4)
2) *In vitro* wheat eIF4G$_{(62-73)}$ pulls down more recombinant human eIF4E than human eIF4G$_{(413-424)}$ does (Fig. 5).
3) Recombinant human 4E-BP1 can compete with either human eIF4G$_{(413-424)}$ or wheat eIF4G$_{(62-73)}$ for binding of recombinant human eIF4E *in vitro* (Fig. 6).

<u>eIF4G peptides specifically inhibit cap dependent translation *in vitro*.</u>

4) wheat eIF4G$_{(62-73)}$ inhibits cap-dependent translation initiation, but not cap-independent translation initiation *in vitro* (Fig. 7).
5) Inhibition of cap dependent translation by eIF4G peptides has not been detected in cultured mammalian cells.
6) No inhibition of general translation by peptides from eIF4G or 4E-BP has been detected in cultured mammalian cells.

<u>eIF4G peptides are cytotoxic *in vitro*.</u>

7) Human eIF4G$_{(413-424)}$-Penetratin exhibits a cytotoxic or cytostatic effect on selected cell lines (HaCaT cells, no effect observed with short treament (<24h with

20μM) but treatment of 60h serum starved cells began to die within 15 minutes of peptide treatment)

8) Human eIF4G$_{(413-424)}$-Penetratin and wheat eIF4G$_{(62-73)}$-Penetratin cause rapid cell death (probably apoptosis) in serum starved cell lines (Fig. 8).

9) Resistance to Human eIF4G$_{(413-424)}$-Penetratin and wheat eIF4G$_{(62-73)}$-Penetratin can result from limited serum treatment (Fig. 9 & 2).

10) Serum induced resistance can be inhibited by pre-treatment with MEK inhibitor PD 098059 (Fig. 9 & 2).

11) Serum induced resistance can be mimicked by the overexpression of a constitutively active MEK/ERK fusion (Fig. 10 & 11).

12) Serum induced resistance of cell lines can be overcome using an increased concentration of peptide (MRC5 cells; 72 h serum-starved cells die rapidly with addition of 10 μM 4G peptides, - cells grown in 10% serum show similar biological effect with 40 μM 4G peptides, - Control peptides (triple Ala substitution) are not cytotoxic at 40 μM

Good SAR

13) Conservation of SAR from wheat and human 4G peptides and human 4E-BP peptides in binding assay, functional cell free assay and cell culture assays.

14) Triple alanine substituted derivatives such as Human eIF4G$_{(413-424)Y416AL421AL422A}$ (see Fig. 8) do not inhibit cap-dependent translation initiation *in vitro*.

15) 4G peptides containing specific single alanine substitutions (such as Human eIF4G$_{(413-424)Y416A}$) partially inhibit cap-dependent translation initiation *in vitro*.

16) Triple alanine substituted derivatives such as Human eIF4G$_{(413-424)Y416AL421AL422A}$–Penetratin do not cause the observed biological effect (apoptosis) in MRC5 cells (Fig. 8).

17) 4G peptides containing specific single alanine substitutions (such as Human eIF4G$_{(413-424)Y416A}$-Penetratin) have an intermediate biological effect on cultured mammalian cells, i.e. reduce rate and extent of cell killing is observed.

* Penetratin is a known cell membrane translocation peptide of sequence RQIKIWFQNRRMKWKK (EP484785B). Description of its synthesis and coupling to other peptides may be found in US Patent 5, ,888.

Expanding on the summary of the results given above;

Fig. 12) A series of biotinylated synthetic peptides (Peptides synthesised by Cyclacel) corresponding to the eIF4E interacting domain of human eIF4G, human BP1 and wheat eIF4G and alanine substituted peptides thereof where tested for their capacity to interact with ^{35}S-Met labelled in vitro translated human eIF4E.

Peptides were coupled to streptavidin coated agarose beads by a N-terminus linked biotin group and washed in PBS/0.2% Tween 3x before being incubated for 1 hour at +4°C with in vitro translated human eIF4E. Beads were washed as above and boiled for 5 min. in SDS loading buffer before the peptide bound proteins were separated on a 10% SDS gel. The bands were visualised by autoradiography.

The three different wild type peptides were shown to interact with human 4E and the H4G Y-A substitution had a lower binding affinity. Scrambled human 4G peptide, the triple alanine human 4G peptide and the triple alanine wheat 4G peptides as well as the single H4G L-A did not interact with 4E.

Fig. 13A, B, C and D) The 4E binding peptides from Fig. 12 were linked with the 16 aa Penetratin carrier sequence of the *Antennapedia* protein and the effect on tissue cultured human fibroblast MRC5 cells were tested after these fusion peptides were added directly to the tissue culture medium (Peptides synthesised by Cyclacel). It is shown that MRC5 cells that have been deprived of FCS for 72 hours die within 15 minutes after treatment with 12 microM of peptides that interact with 4E. Cell death is scored by analysing 4 different fields of approximately 200 cells each in 4 parallel experiments in which living and dead cells are counted.

Fig. 14) In the presence of 10% FCS cells are resistant to treatment with 20 microM of the peptides, only if the cells are serum deprived for 72 hours will they die (more then 85%) within 15 minutes after the peptide have been applied. If serum deprived cells (72h) are pre-treated with 10% FCS or with 20nM PMA(phorbol ester) for 15 minutes before the peptides are added the cells survive peptide treatment (60-70%).

Further more, if the serum deprived cells are instead pre-treated with the MAPK inhibitor PD098059 for 1 hour before 10% FCS is added about 80-90% of the cells will die. This result shows that cell death is linked to a genetic program and that the cells can be rescued from peptide induced death by addition of FCS or PMA. It is also suggested by the speed with which the cells die after peptide treatment and the rapid rescue by FCS or PMA and the effect of the MAPK inhibitor, that the effect of the peptides on cell death is dependent on secondary modifications in the cells.

Fig. 15) Serum deprived cells were treated with the general translation inhibitors Cyclohexamide or Pactamycin at indicated concentrations or the H4G peptide in the presence of 35S-Met for 30 minutes. Cells were lysed and the amount of translation was estimated by counting incorporated 35S-Met in precipitated protein fractions. As expected, the peptide treated cells do not incorporate 35S-Met and they die. However, general translation inhibitors block translation but they do not kill the cells.

In another experiment, cells that have been serum deprived for 72hours and pre-treated with general translation inhibitors are shown to be just as susceptible to cell death (85%) as cells not treated with translation inhibitors. This strongly indicates that the effect of cell killing by the peptides is not mediated by inhibition of translation and is therefore not mediated by a translation product. This observation is very surprising and novel.

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2. Hentze, M. eIF4G: a multipurpose ribosome adapter? *Science* **275**, 500-1 (1997).
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14. Fukuchi-Shimogori, T. *et al.* Malignant transformation by overproduction of translation initiation factor eIF4G. *Cancer Res* **57**, 5041-4 (1997).

15. Polunovsky, V. *et al.* Translational control of programmed cell death: eukaryotic translation initiation factor 4E blocks apoptosis in growth-factor-restricted fibroblasts with physiologically expressed or deregulated Myc. *Mol Cell Biol* **16**, 6573-81 (1996).

16. Fletcher, C. *et al.* 4E binding proteins inhibit the translation factor eIF4E without folded structure. *Biochemistry* **37**, 9-15 (1998).

Figure 1

Interaction of eIF4G and eIF4E

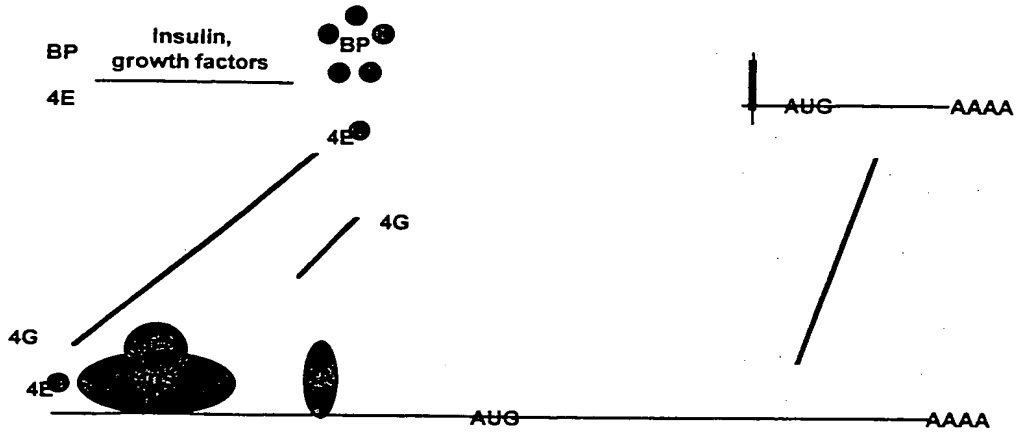

Figure 2

Interaction of eIF4G and eIF4E

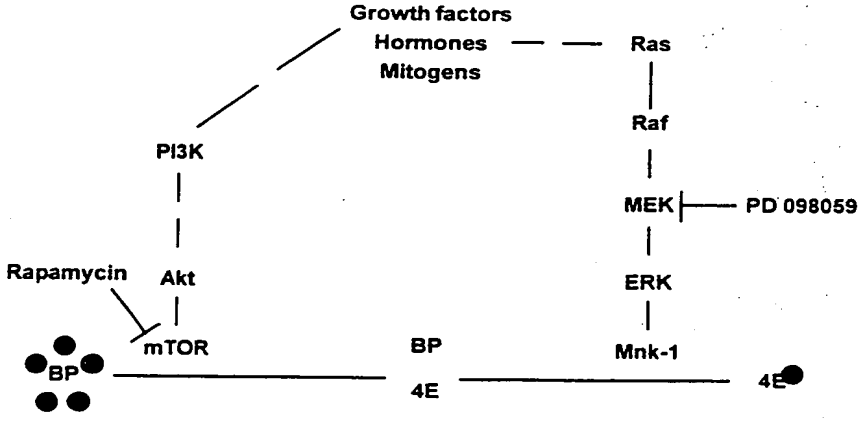

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Figure 3

Interaction of eIF4G and eIF4E

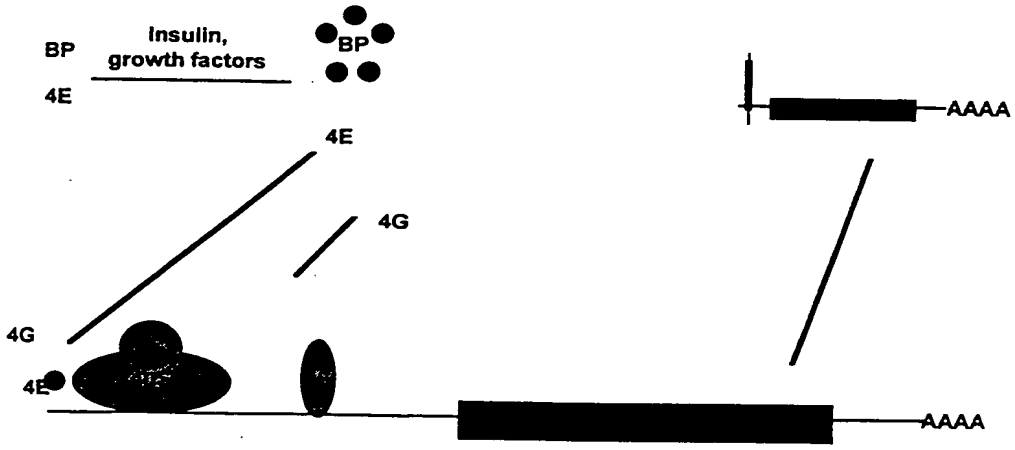
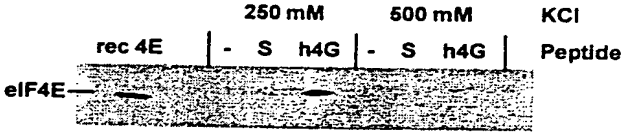


Figure 4

Human eIF4G-Penetratin binds eIF4E



h4g human eIF4G B β KKRYDREFLLGFAARQIKIWFQNRRMKWKK

S scrambled eIF4G B β FDLKFALGRYRAEKRQIKIWFQNRRMKWKK

\- no peptide

rec 4E recombinant human eIF4E

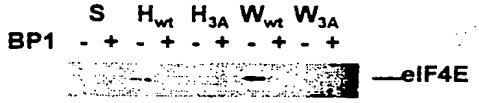
Figure 5

...but Wheat eIF4G binds better

H	human eIF4G	B	β	β	K	K	R	Y	D	R	E	F	L	L	G	F	413-424
Y	yeast eIF4G	B	β	β	K	Y	T	Y	G	P	T	F	L	L	Q	F	449-460
W	wheat eIF4G	B	β	β	R	V	R	Y	S	R	D	Q	L	L	D	L	62-73
1	human 4E-BP1	B	β	β	R	I	I	Y	D	R	K	F	L	M	E	U	51-62
2	human 4E-BP2	B	β	β	R	I	I	Y	D	R	K	F	L	L	D	R	51-62
S	scrambled eIF4G																

Figure 6

Human 4E-BP1 competes with 4G peptides for binding of eIF4E



	4G Peptide	Sequence
H_{wt}	hu $4G_{(413-424)}$	K K R Y D R E F L L G F A A
H_{3A}	hu $4G_{(413-424)YALALA}$	K K R A D R E F A A G F A A
W_{wt}	wh $4G_{(62-73)}$	R V R Y S R D Q L L D L A A
W_{3A}	wh $4G_{(62-73)YALALA}$	R V R A S R D Q A A D L A A
S	scrambled hu 4G	F D L K F A L G R Y R A E K

all peptides biotinylated and linked to Penetratin™

Figure 7

Wheat eIF4G$_{(62-73)}$ inhibits Cap-dependent translation initiation

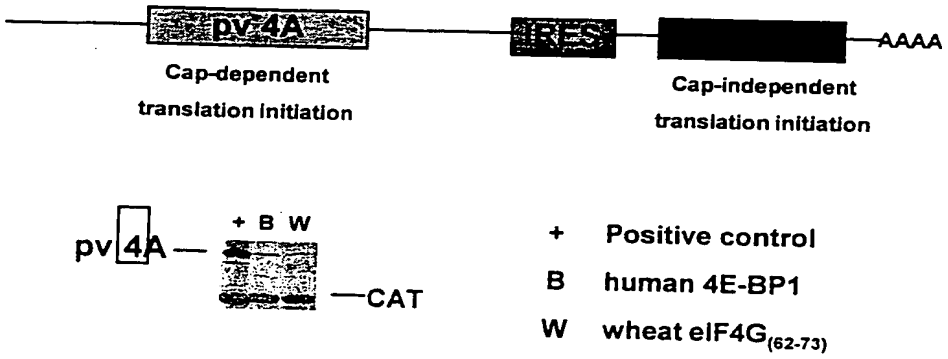


Figure 8

Induction of apoptosis by eIF4G peptides

Peptide	Concentration			
	3 μM	6 μM	9 μM	12 μM
hu 4G$_{(413-424)}$	+	++	+++	+++
hu 4G$_{(413-424)Y416AL421AL422A}$	-	-	-	-
wh 4G$_{(62-73)}$	-/+	+	++	+++
wh 4G$_{(62-73)Y65AL70AL71A}$	-	-	-	-
scrambled hu 4G	-	-	-	-

MRC5 cells, 72 h serum-free growth, all peptides biotinylated and linked to Penetratin™

Figure 9

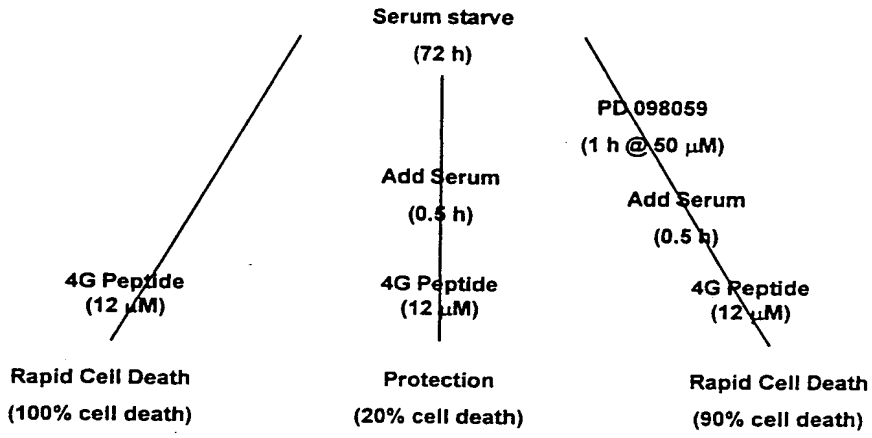


Figure 10

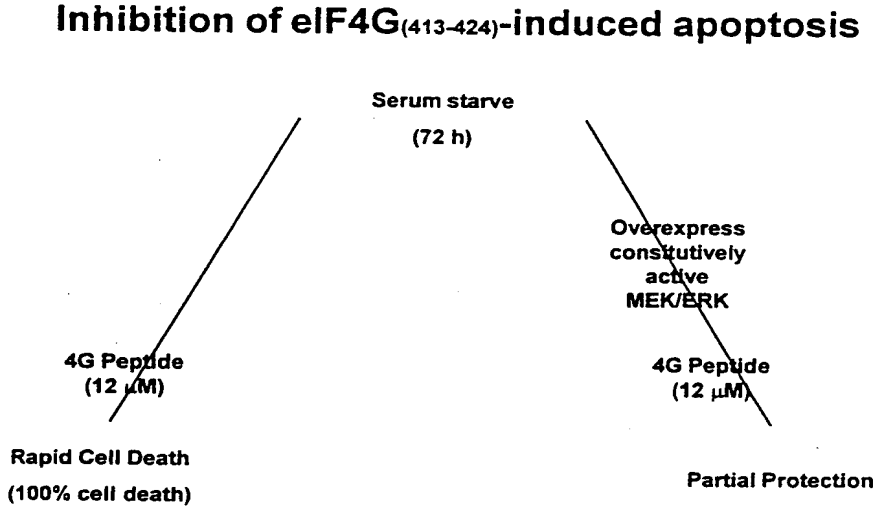
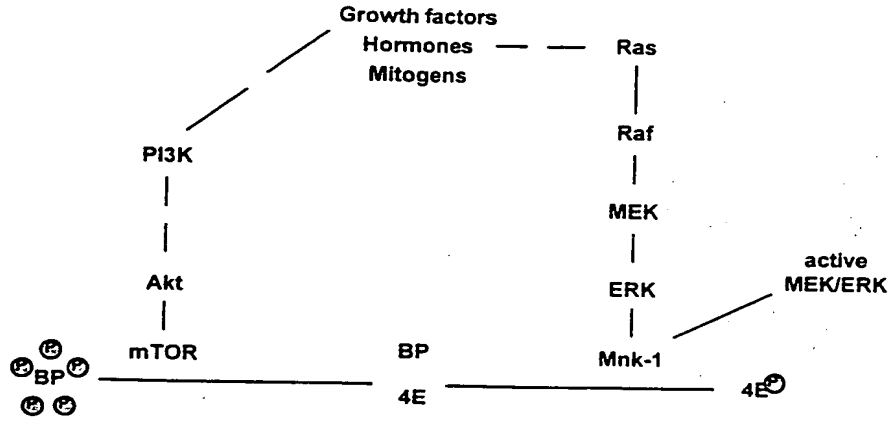


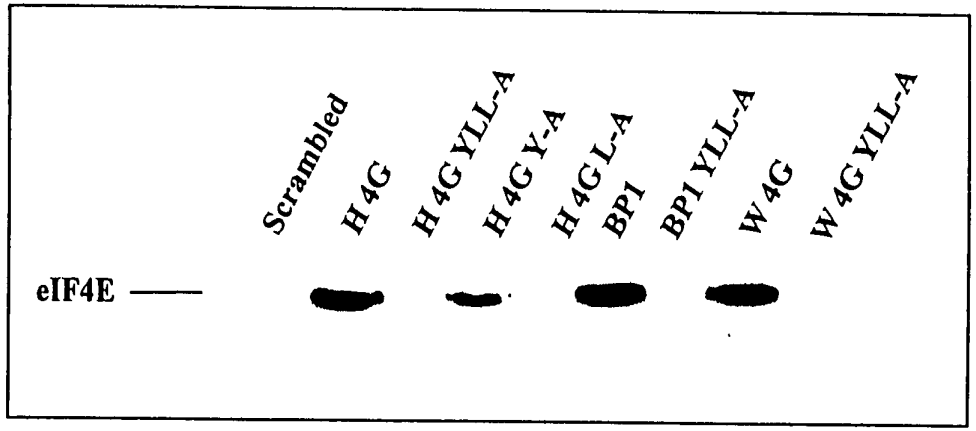
Figure 11

Interaction of eIF4G and eIF4E



In vitro binding analysis of eIF4E binding peptides

Hu 4G	Human eIF4G(413-424) Peptide Wild Type	KKRYDREFLLGF
Hu 4G Y11-A	Human eIF4G Peptide (413-424) Y416A L421A L422A	KKRADREFAAGF
Hu 4G Y-A	Human eIF4G Peptide (413-424)Y416A	KKRADREFLLGF
Hu 4G L-A	Human eIF4G Peptide (413-424)L421A	KKRYDREFALGF
W4G	Wheat eIF4G Peptide (62-73)Wild Type	RVRYSRDQLLDL
W4G YLL-A	Wheat eIF4G Peptide (62-73)Y65A, L70A, L71A	RVRASRDQAADL
BP1	Human 4E-BP1 (37-48) peptide Wild Type	RIIYDRKFLLDR
BP! YLL-A	Human 4E-BP1 peptide (37-48) Y40A, L45A, L46A.	RIIADRKFAADR



Streptavidin sepharose-biotinylated peptide pulldown

Figure 12

Human-4G peptide causes cell death in MRC5 cells

Human eIF4G(413-424) Peptide Wild Type	KKRYDREFLLGF
Human eIF4G Peptide (413-424) Y416A L421A L422A	KKRADREFAAGF

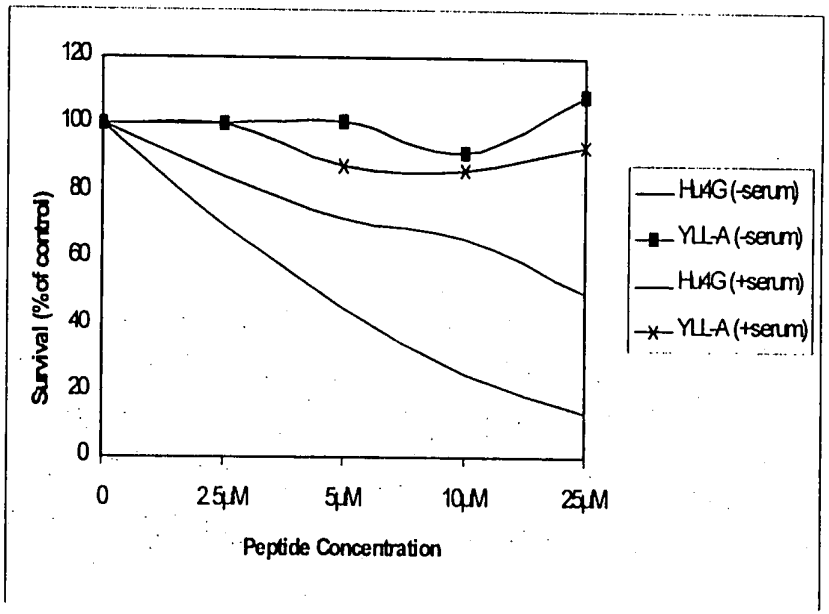
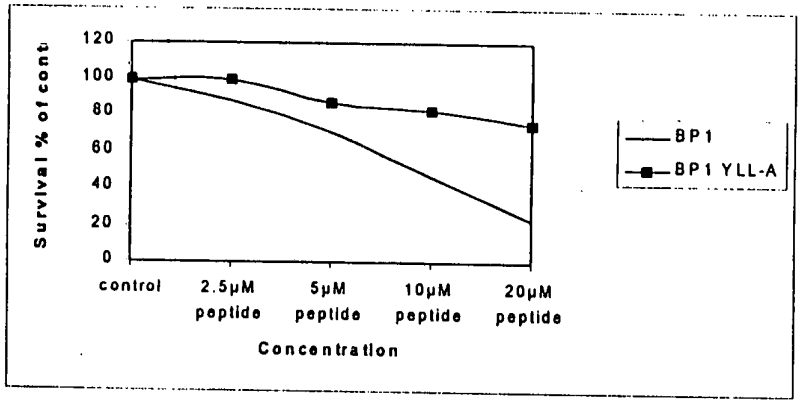


Figure 13a

Effect of human 4E-BP1 peptide on cell survival

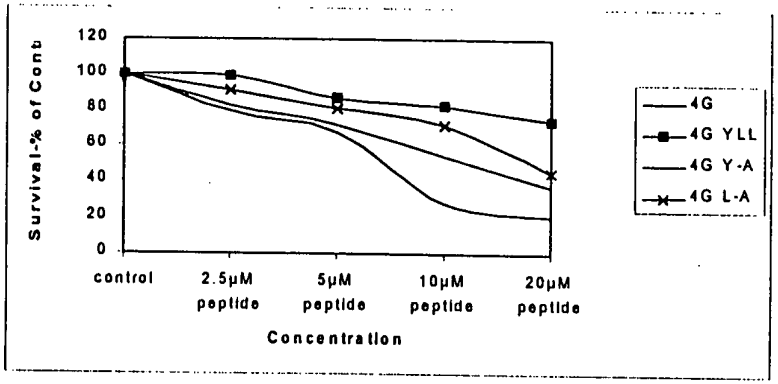


BP1	Human 4E-BP1 (37-48) peptide Wild Type	RIIYDRKFLLDR
BP1 YLL-A	Human 4E-BP1 peptide (37-48) Y40A, L45A, L46A.	RIIADRKFAADR

Control **BP1** **BP1 YLL-A**

Figure 13b

Effect of human-4G peptide and alanine substitutions cell survival



Hu 4G	Human eIF4G(413-424) Peptide Wild Type	KKRYDREFLLGF
Hu 4G Yll-A	Human eIF4G Peptide (413-424) Y416A L421A L422A	KKRADREFAAGF
Hu 4G Y-A	Human eIF4G Peptide (413-424)Y416A	KKRADREFLLGF
Hu 4G L-A	Human eIF4G Peptide (413-424)L421A	KKRYDREFALGF

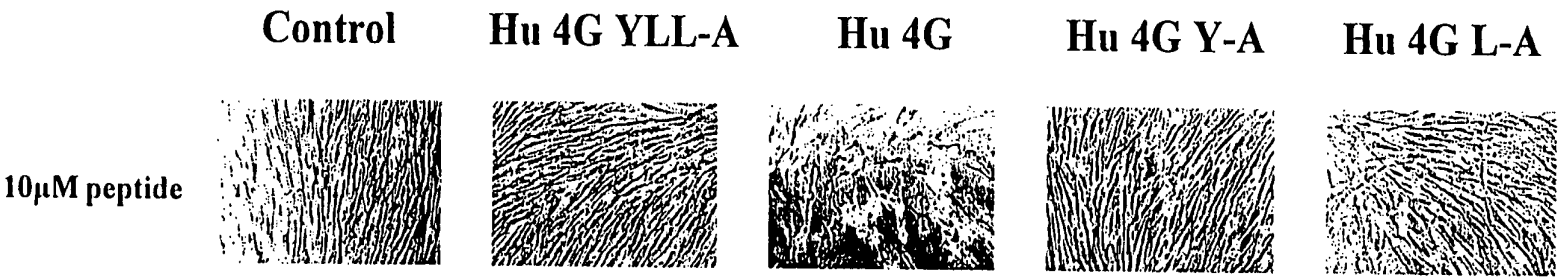
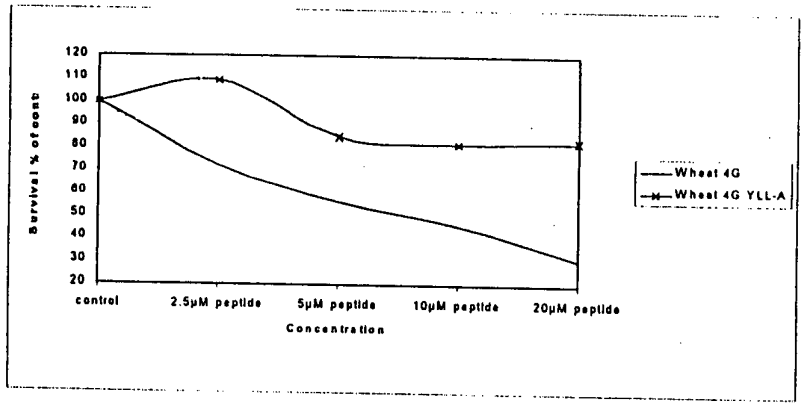


Figure 13c

Effect of wheat 4G peptide on cell survival



W4G	Wheat eIF4G Peptide (62-73)Wild Type	RVRYSRDQLLDL
W4G YLL-A	Wheat eIF4G Peptide (62-73)Y65A, L70A, L71A	RVRASRDQAADL

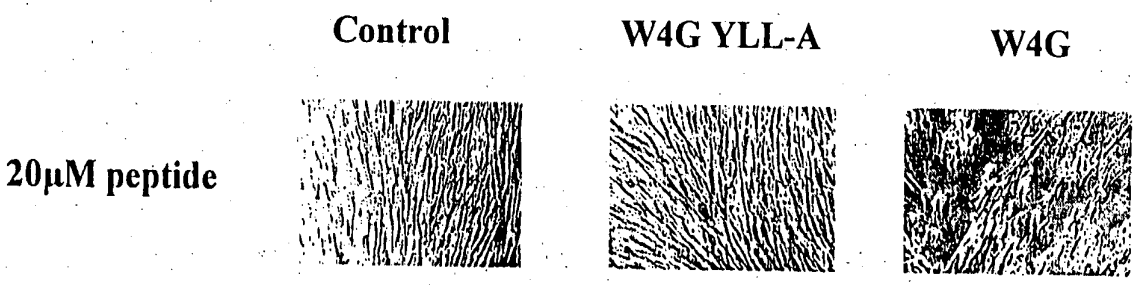


Figure 13d

Acute activation of MAP kinase protects cells from 4E-binding peptide induced cell death

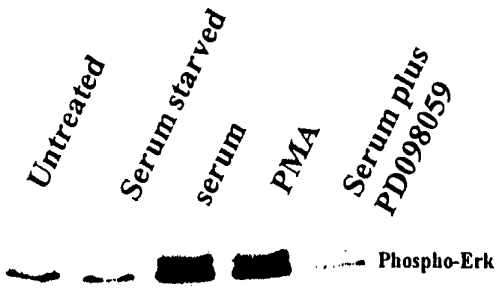


Figure 14

4E binding peptide induced cell death is not through t general inhibition of mRNA translation

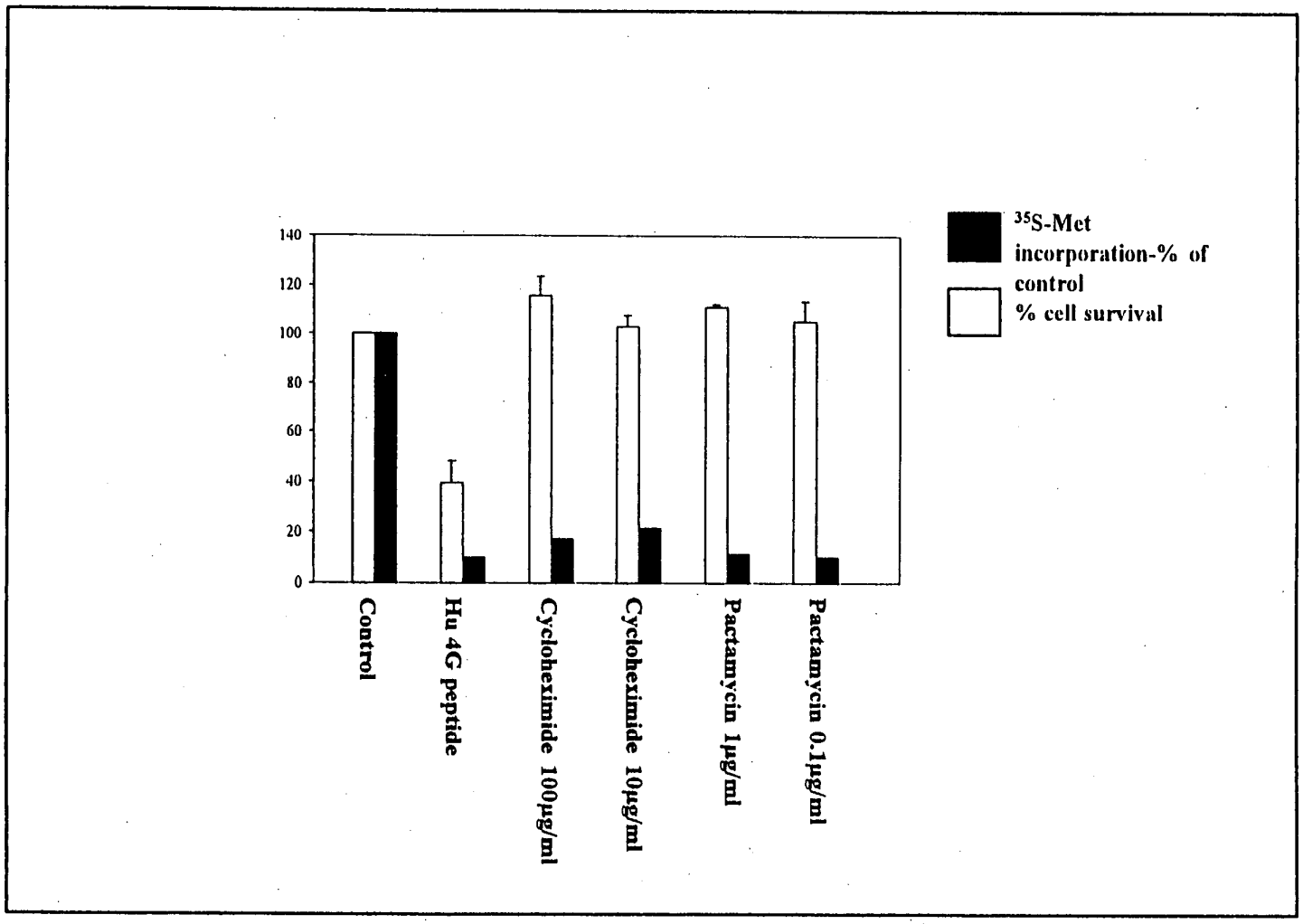


Figure 15

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Cruickshank & Fairweather